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# Food consumption pattern and baseline susceptibility to *Bacillus thuringiensis* Berliner var. *tenebrionis* in Colorado potato beetle larvae

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## ABSTRACT

The food consumption pattern of Colorado potato beetle larvae revealed that the food intake increased exponentially with larval age, with the penultimate and ultimate instars larvae consuming18 and 73% of the total, respectively. Different larval stages of Colorado potato beetle were treated by different doses of *Bcillus thuringiensis* Berliner var. *tenebrionis* under laboratory conditions to determine larval susceptibility to this bioinsecticide. The results indicated that in terms of $LD_{50}$ values younger larvae were more susceptible to the *B. thuringiensis* than older instars. $LD_{50}$ values estimated from the collected data indicated a different pattern of susceptibility of larvae to *B.t.* for different larval stages, as evidenced by non overlapping confidence intervals of relative median potency estimates The $LT_{50}$ values showed that the speed of larval death depends on the larval age and *B. thuringiensis* dose. It was concluded that, if the pattern of food consumption and susceptibility in the present study was used as a model of larval food intake and susceptibility under natural conditions, the larvae must be killed prior to penultimate instar.

**Key words:** feeding activity, frass production, bioassay, lethal dose ($LD_{50}$), *Bacillus thuringiensis*

## INTRODUCTION

*Leptinotarsa decemlineata* (Say) is the most devastating defoliator of potato plants worldwide (Zhao *et al*., 2000; Igrc *et al*., 2006). Larvae and adults feed on foliage and if potato plants left untreated, complete defoliation of plants is possible. Crop rotation and non-chemical tactics such as biological control for regulating populations are useful in some situations, however, management of this pest has relied almost totally on synthetic chemical insecticides. Such reliance on insecticides for control has resulted in insecticide resistance to most insecticide chemical groups (Bishop & Grafius, 1996; Stewart *et al.*, 1997; Scott *et al*., 2003) and recently to commercialized new neonicotinoids (Mota-Sanchez *et al.*, 2006).

The effectiveness of application of a microbial insecticide such as *Bacillus thuringiensis* (hereafter referred to as *B.t.)* largely depends on a number of factors such as concentration, droplet deposition on the target plant (Svestka & Vankova, 1984) and post spray environmental factors particularly rainfall and sun light. Since *B.t*. must be ingested to be effective the dose ingested by a target insect is a crucial factor. To ensure satisfactory control of an insect, a lethal dose of *B.t*. must be consumed by each insect before degradation or washed away off by rain occurs. Therefore, the knowledge of food consumption and lethal does are fundamental to

predict more accurately the time and quantity of *B.t*. to be applied over per unit area for effective control. Scriber and Slansky (1981) stated that the total amount of food eaten by an immature insect increases during the 3rd and 4th instars. As food consumption of immature insects is age-dependent, the age is another important parameter to be considered in *B.t*. application. Ideally a comprehensive understanding of the contribution of each instar to defoliation is essential for optimizing the efficacy of *B.t*. as a microbial pest control agent.

Over the last 40 years, evaluation of the efficacy of different preparations of *B.t*. on different insects has been based mainly on bioassays either by incorporating *B.t*. into larval diet or by surface contamination (Morris, 1988; Dias *et al*., 2005; Crickmore, 2006). Although the fundamental rule and significance of such methods in the development of more effective preparations of *B.t.* is well known, they also possess some shortcomings. The major drawback is that it is unknown how much *B.t*. is ingested by each individual. Therefore, it was necessary to employ a technique for administration of *B.t*. in which the same quantity was consumed by each larva over the same period of time. The technique developed involved voluntary ingestion of the entire dose within a very short period of time using natural food (leaf disk) as a carrier of *B.t*. This technique used to evaluate the larval susceptibility in terms of $LD_{50}$ to *B.t*. infection.

There is no record of *B. t.* application on potato in Zanjan province so far. On the other hand, current wisdom agreed that, it is essential to have some estimates of changes in susceptibility level of Colorado potato beetle where a particular insecticide could be applied in future. Such an estimate requires initial baseline data of pest population susceptibility and thereafter comparisons can be feasible. Little literature is available concerning the toxicology of *B.t*. preparations in terms of $LD_{50}$ and larval food in take pattern of Colorado potato beetle. The present investigation was therefore designed to provide some baseline $LD_{50}$ data and food consumption pattern for Colorado potato beetle larvae.

## MATERIALS AND METHODS

**Insects:**

Colorado potato beetles were collected by hand from April to July 2009 from four different untreated potato fields in Zanjan (36.40° N 48.31° E) a town in Iran, and reared on greenhouse grown potato foliages; maintained at 27 ± 3°C, relative humidity of 50 ± 10%, and a photoperiod of 16:8 (L: D) h. The fields were in a major agricultural area with high crop diversity.

**Insecticide:**

The *Bacillus thuringiensist* var. *tenebreonis* (Btt) preparation was obtained from Mehr Asia Biotechnology Co. This was a commercial SL formulation containing spores and crystals (Count Fertilizer Unit = $10^8$/ ml).

**Bioassay:**

For each larval stage on the basis of preliminary tests, five concentrations of *B.t.* were tested to produce ≈ 25-75% mortality at the lowest and the highest doses, respectively (Robertson *et al*., 2007). In each trial, untreated group served as a control. Fresh preparations of *B.t.* were made for each bioassay. At the time of bioassays larvae were selected for uniformity in size and only vigorous insects were used. The treatments were replicated four times on four different days. In each bioassay, results of all replicates were pooled. The highest concentration, 20 mg/ml, was prepared in distilled water. Then serial 1: 2 dilutions were made to produce the

6 required concentrations ranging from 0.63-20 mg/ml. For each larval age class 5 appropriate concentrations were used.

**Food consumption pattern of larvae:**

To determine the pattern of food consumption by different larval stages, twenty neonate (up to 24 h) old larvae were selected at random from the stock culture and placed in 280 $cm^3$ translucent round box approximately 63.6 × 4.4 cm on a green house grown fresh potato leaf. Larvae were kept in the rearing room at 27 ± 3°C, relative humidity of 50 ± 10%, and a photoperiod of 16:8 (L: D) h and more than enough fresh leaves were provided as needed. The frass produced by the larvae at the termination of first instar was collected. For second, third, and fourth instars age groups, the conditions for maintaining and monitoring the larvae all were the same as those in the first instar larvae described above. Based on Li (2005) report, frass production was used as an indicator of feeding activity of *L. decemlineata* larvae. Fresh frass was oven-dried at 40°C for 4 d. Dried frass was weighed to the nearest 0.1 mg, by using a Mettler analytical balance (Mettler Instrumente, Zurich, Switzerland). The dried frass weight was expressed in milligrams per larva (mg/larval age) and calculated by dividing the total frass weight by the total larvae for each age group. The experiment was replicated four times and results of all replicates were pooled.

**Larval mortality rate**

**First instar larvae:**

Leaf disks (30-mm diameter) were cut from fully expanded green house grown potato plants and dipped in five concentrations of *B.t.* (10, 5, 2.5, 1.25 and 0.63 mg /L) for 30 s. Leaf disk treated with water served as a control treatment. After 5 min.-air-drying, each leaf disk was placed into 35-mm diameter plastic cups containing 10 ml of solidified 2% agar solution. Fifteen first instars larvae (up to 24 h) were placed into each plastic cup, sealed with parafilm and maintained at 27 ± 3°C, relative humidity of 50 ± 10%, and a photoperiod of 16:8 (L: D) h. The experiment was replicated four times on four different days.

**Second and third instars larvae:**

Three droplets of 2% agar solutions were deposited in each 8 × 100 mm glass tubes and left at room temperature to solidify. Larvae from second and third instars were selected at random from the stock culture. One larva was placed in each tube. Spoons shaped of 4 $mm^2$ were punched out of young green house grown potato leaves with a bended number 2 cork borer. The size of leaf disk was such that third instars larvae could consume the entire disk with only 4 or 5 bursts of feeding within a very short period. For each dose of *B.t.* 2 µl of the suspension was administrated to the centre of each leaf disk with a hand operated Arnold microapplicator. After the evaporation of solvent (water), a single disk was placed in each glass tube and the tubes sealed with cotton wool. Leaf disks treated with water only were fed to control larvae. The tubes were kept in the rearing room under standard conditions. In all cases the larvae which failed to consume the entire disk within 5 hours were discarded. After 5 hours those which consumed the whole treated disk were transferred to a 35 ml translucent container with fresh untreated leaves and placed in the rearing room. The larvae were provided with fresh, untreated leaves of potato as needed. Since control larvae entered pupal stage after 7 days, mortality was recorded at the end of 7 days. For each concentration 4 replicates of 15 larvae from either the second or third instars were tested.

**Lethal Time:**

During the larval susceptibility experiments, larval mortality was recorded daily. The mortality data for each bacterium dose was analyzed with a complementary log-log model (Robertson *et al.,* 2007). The relative median potency was used to determine significance differences in the $LT_{50}$ values between the two *B.t.* doses within an instar at the $\alpha = 0.05$ level.

**Data analysis:**

Mortality data from all bioassays were analyzed with SPSS software (Norůsis, 2008) assuming the probit model. Median lethal doses ($LD_{50}$s) and their corresponding 95% CI were estimated. The values and significance of $\chi^2$ and the 95% CI for potency ratios were determined. Lethal time fifty value ($LT_{50}$) was estimated using complementary log-log model. Parallel regression lines were also compared using overlapping confidence limits ($P \leq 0.05$) of relative potencies as the criterion to detect significant differences in mortality time.

## RESULTS AND DISCUSSION

**Food consumption pattern of larvae:**

Monitoring frass production as an indicator of food consumption revealed the importance of penultimate and ultimate instars which consumed more than 91% of the total larval food intake, whereas first and second instars larvae produced only 3 and 6% of total frass production. Ultimate and penultimate larvae therefore could contribute most to plant defoliation (Fig. 1). In addition, Fig.1 shows a significant difference among frass production of the different larval instars. Therefore, if the pattern of food consumption in the present study is used as a model of larval food intake under natural conditions, the larvae must be killed prior to penultimate instar. To explain the relationship between the changes in larval food consumption, a number of regression curves were fitted to the data. The best description for the relationship between frass production and larval age was obtained by the equation:

$$Y = 34.5 - 42.8\ X + 13\ X^2$$

Where Y = frass weight (mg), X = larval age (instar).

The equation has the potential to describe more than 97% of the relationship and allows the prediction of the relative larval food intake.

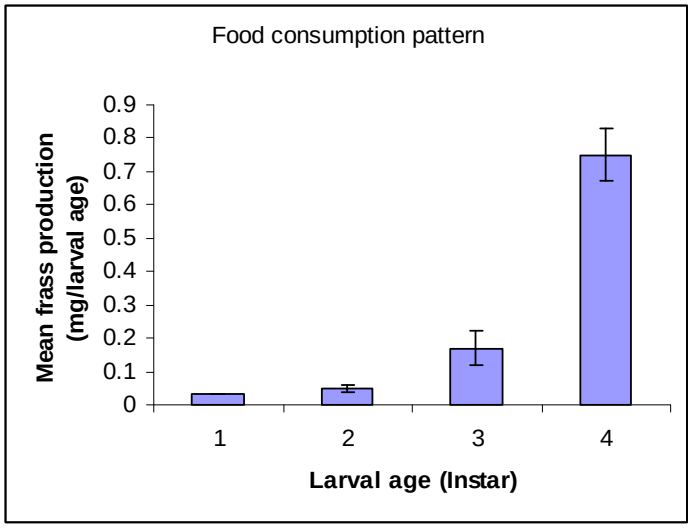


Fig. 1: Quantitative pattern of food consumption by *Leptinotarsa decemlineata* larvae.
A significant difference among the means ± SE of frass production for various larval age is detected.

## Larval susceptibility:

$LD_{50}$ values estimated from the collected data indicated a different pattern of susceptibility of larvae to *B.t.* for three different larval stages, as evidenced by non-overlapping confidence intervals of relative median potency estimates (Table 1). The slopes of the dose-response ranging 1.35-1.79 which are relatively steep and mortality rates between the highest and lowest were low (Table 1). This is an indication of homogeneity of the population in samples. Therefore, steps should be taken to promote population stability through integration of all IPM program tools such as augmentation of susceptible individuals in population. Parallelism test revealed a non significant difference between dose-response lines ($\chi^2 = 2.58$; $P = 0.28$). This could be an indication of target site(s) similarity for all populations tested (Robertson *et al.*, 2007). Estimation of relative median potency for population from first instar versus second and third instars larvae yielded 0.7 – 1.36, and 0.32-0.71 values for lower and upper confidence intervals, of second and third instars larvae, respectively. Therefore, a non-significant difference between the susceptibility of first and second instars larvae was detected. Whereas, a significant difference between susceptibility of first and third instars larvae was observed. Likewise, a significant difference between the susceptibility of second and third instars larvae was detected.

Table 1: Toxicity of *Bacillus thuringiensis* to first, second and third instars of *Leptinotarsa decemlineata* larvae.

| Toxicity value | Toxicity to larvae μ g / larva determined for | | | | | |
|---|---|---|---|---|---|---|
| | First instar | | Second instar | | Third instar | |
| | LD50 | LD90 | LD50 | LD90 | LD50 | LD90 |
| Lethal dose | 2.450 | 21.86 | 2.49 | 12.93 | 5.07 | 39.11 |
| Upper 95% CI | 3.21 | 53.81 | 3.07 | 22.32 | 6.55 | 86.76 |
| Lower 95%CI | 1.86 | 12.88 | 2.01 | 9.02 | 3.92 | 24.13 |
| Slope ± SE | 1.35 ± 0.18 | | 1.79 ± 0.21 | | 1.44 ± 0.20 | |
| Number of insects tested | 1080 | | 1080 | | 1080 | |
| $\chi^2$ | $1.63^{ns}$ | | $4.29^{ns}$ | | $1.71^{ns}$ | |
| *P* | 0.65 | | 0.23 | | 0.61 | |

Results of four replicates per treatment were pooled.
*P* value ≥ 0.05 indicates no significant deviation between the observed and expected regression lines in a log-probit analysis.

## Lethal time:

In terms of $LT_{50}$ the cumulative control mortality for second and third instars larvae were 3.89 and 4.86 d respectively (Table 2). Within an instar, larvae died at the highest dose of 20 mg/L. Most tested larvae died between 3 and 6 d after treatment. No additional larval mortality was observed 6 d post treatment for both second and third instars at the highest concentration. Within a given dose, it took much longer to kill the same percentage of older larvae than younger one. In terms of $LT_{90}$ values it took ≈ 6.22 and 7.99 d for second and third instars, respectively. The knowledge of $LT_{90}$ as discriminating lethal time is an important issue from control measures stand point of view for practitioners under field conditions. Parallelism test revealed a non significant difference between dose-response lines ($\chi^2 = 0.21$; $P = 0.65$). Estimation of

relative median potency for second versus third instars larvae yielded 0.81, 0.67 and 0.93 values for estimate, lower and upper confidence intervals, respectively.

Therefore, a significant difference between the lethal time fifty of second and third instars larvae was secured.

The amount of food consumption of an insect is a fundamental factor in the acquisition of an effective dose of an orally acquired insecticide. The acquisition of a lethal dose is a function of food consumption rate and insecticide concentration.

The importance of quantitative information on food consumption of phytophagous pest insects has been stressed earlier (Parra & Kogan, 1981). Although the results of food consumption on green house grown host plants under laboratory conditions cannot be directly extrapolated to the field, it can be useful in predicting potential damage. Monitoring daily food consumption revealed the importance of penultimate and ultimate instars which consumed more than 91% of the total larval food intake. Ultimate and penultimate larvae therefore contribute most to plant defoliation. Therefore if the application of *B.t*. is to provide good crop protection, the larvae must be killed prior to the penultimate instar. The criterion for making decisions on the time of application of *B.t*. against larvae should be on the basis of the amount crop loss which can be tolerated. For instance, potato plants can withstand 10-20% defoliation without noticeable reduction in the yield (Hare, 1990). Therefore, application of *B.t*. may be delayed until initiation of penultimate instar emergence.

Table 2: Over time mortality of *Leptinotarsa decemlineata* larvae fed on potato leaves treated with 20 µg/L of *Bacillus thuringiensis*.

| Time mortality value | Time mortality to larvae | | | |
|---|---|---|---|---|
| | Second instar | | Third instar | |
| | LT / d determined for | | | |
| | LT50 | LT90 | LT50 | LT90 |
| Lethal time | 3.89 | 6.22 | 4.86 | 7.99 |
| Upper 95% CI[a] | 4.29 | 7.72 | 5.40 | 11.05 |
| Lower 95%CI | 3.41 | 5.51 | 4.34 | 6.82 |
| Slope ± SE | 6.28 ± 1.11 | | 5.93 ± 1.26 | |
| $\chi^{2}$ [b] | 1.48 [ns] | | 3.75 [ns] | |
| *P* | 0.68 | | 0.29 | |

[a] 95% confidence intervals of the $LT_{50}$ value.
[b] No $\chi^2$ was significant at the $\alpha = 0.05$ level.

Results from this study demonstrated that higher larval mortality of *L. decemlineata* was associated with younger larvae at a given *B.t.* dose (Table 1), suggesting that early instars were more susceptible to the *B.t.* than older larvae. Higher susceptibility of younger larvae to their respective microbial insecticides than older instars have been previously reported (Milks *et al*., 1998; Duan & Otvos, 2001). Data from this study on larval susceptibility to the *B.t.* may have significance in terms of field operational sprays of the *B.t.* against *L. decemlineata*. Based on the fact that younger larvae were more susceptible to the *B.t*. than older instars, field sprays should target early instars to maximize control efficacy and minimize foliage damage because older larvae become less susceptible to the *B.t*. and consume more foliage.

With respect to $LC_{50}$ values the inverse relationship between larval susceptibility to *B.t*. and biomass has been reported by numerous authors (e.g., Hornby & Gardner,

1987). However, despite such extensive information on $LC_{50}$ of different preparations of *B.t.*, little literature is available with regard to $LD_{50}$ of this microbial insecticide. Positive relationship between $LD_{50}$ values and larval instars (weight) suggests the application of *B.t.* should be directed towards younger larvae. If the pattern of food consumption in the present study is used as a model of larval food intake under natural conditions, the larvae must be killed prior to penultimate instar. In such circumstances application of the estimated lethal dose for penultimate instar larvae will also suppress the younger larvae. Thus, the information on food consumption and lethal dose are need to time of *B.t.* application.

Based on the estimated $LD_{90}$ values, the mean toxicity of the *B.t.* to tested age groups could be rated in the following order: second instar > first instar > third instar. This implies that, second instar larvae is more appropriate age group for estimation of discriminating dose which is an important issue from control measures stand point of view.

## CONCLUSION

Managing resistance of *L. decemlineata* requires comprehensive knowledge of levels and distribution of resistance that exist within the potato-growing region. Determining the baseline susceptibility levels in terms of $LD_{50}$ and extent of resistance to insecticide in field populations of Colorado potato beetle is an essential first step toward developing its resistance management strategies. Determination of baseline susceptibility data in conjunction with food consumption activity are indispensable components in planning control operations. It is concluded that, if the pattern of food consumption and susceptibility in the present study is used as a model of larval food intake and susceptibility under natural conditions, the larvae must be killed prior to penultimate instar.

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